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L1

Enhancing the thermal stability of avidin

FIELD OF THE INVENTION

Avidin is a protein occurring in egg white, where it constitutes approximately 0.2% of the total protein. It has the property of combining firmly with biotin and rendering it unavailable to organisms, since proteolytic enzymes do not destroy the avidin-biotin complex. Avidin loses its ability to combine with biotin when subjected to heat.

The present invention relates to a method for enhancing the thermal stability of avidin by introducing disulphide bridges between its subunits and to the thermostable avidin mutants produced. These new avidin mutants will find use in applications where extreme stability or minimal leakage of subunits is required.

BACKGROUND OF THE INVENTION

Chicken avidin and bacterial streptavidin are widely utilized proteins in many life science applications ranging from purification techniques to modern diagnostics and targeted drug delivery. This methodology, known as (strept)avidin-biotin technology, relies on the extremely tight and specific affinity (Kd ~ 10^{-15} M) between (strept)avidin and biotin.

Avidin and streptavidin are exceptionally stable proteins consisting of homotetrameric up-and-down β-barrels, which upon biotin binding become even more stable. Transition midpoints of heat denaturation (T_m), analyzed by differential scanning calorimetry (DSC), have shown that avidin is more stable than streptavidin in both the absence and presence of biotin (Gonzalez, M., Argarana, C.E., & Fidelio, G.D., Biomol. Eng. 16, 67-72 (1999). A possible reason for this may be the intramonomeric disulphide bridge found in each avidin monomer. Wild-type (wt) avidin has a high isoelectric point and it is glycosylated, which properties may cause unspecific binding in some applications. It has been shown, that these unwanted properties can be abolished without markedly affecting the tight biotin-binding affinity and the stability characteristics of avidin (Marttila, A.T. et al., FEBS Lett 467 (2000), 31-6)..

The avidin (and streptavidin) tetramer is actually a dimer of two dimers. The monomers that form the tetramer interact with each other in a symmetrical manner and form the three types of monomer-monomer interactions described in detail by

Livnah (Livnah, O., Bayer, E.A., Wilcheck, M., & Sussman, J.L., Proceedings of The National Academy of Sciences of the United States of America 90 (1993), 5076-5080). The interaction between monomers 1-4 (and the equivalent 2-3) is the strongest, while the interaction between monomers 1-3 (and 2-4) is the weakest. The intensity of the interaction between monomers 1-2 (and 3-4) is also relatively weak, but important because tryptophan 110 in avidin (Trp120 in streptavidin) from subunit 1 participates in biotin binding at the binding-site of subunit 2, forming the function-related monomer-monomer interface.

A streptavidin mutant with enhanced stability characteristics when compared to those of the wild-type streptavidin has been reported previously (Reznik, G.O. et al., Nat Biotechnol 14 (1996), 1007-11). The improved stability was achieved by addition of two intermonomeric disulphide bridges to the streptavidin tetramer, one between monomers 1-3 and the other between monomers 2-4, by changing histidine residue 127 to cysteine. In avidin these interfaces are similar, and histidine residue 127 of streptavidin is analogous to isoleusine residue 117 of avidin.

SUMMARY OF THE INVENTION

The object of this invention is to provide a stabilized chicken avidin. This was achieved by introducing disulphide bridges between its subunits. These covalent bonds had no major effects on the biotin-binding properties of the respective mutants. Moreover, one of the mutants maintained its tetrameric integrity even in denaturing conditions. These new avidin forms have native → denatured transition midpoints (T_m) close to 100 °C in the absence of biotin. Furthermore, we showed that the intramonomeric disulphide bridges found in wild-type avidin have effects on its stability. A mutant form, in which this bridge was removed, had a lower Tm in the absence of biotin than wild-type avidin, but showed comparable stability in the presence of biotin.

In the present invention the stability of avidin was improved through the addition of intermonomeric disulphide bridges by changing isoleucine residue 117 to cysteine. In addition two supplementary mutants were produced where even more intermonomeric disulphide bridges were introduced. A cysteineless avidin version was also constructed, where the intramonomeric disulphide bridges of wild-type avidin were removed. The stability characteristics of these mutants were studied and compared with those of wild-type avidin and streptavidin.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A shows schematic representation of the mutants Avd-ci, Avd-cci and Avd-ccci, displaying the intermonomeric disulphide bridges.

FIG. 1B shows non-reducing SDS-PAGE analysis of avidin (Avd) and avidin mutants Avd-ci, Avd-cci and Avd-ccci. Samples were boiled for 15 minutes in SDS-PAGE sample buffer (without β-mercaptoethanol) and the gel was stained with Comassie brilliant blue. Wild-type avidin (Avd) is mainly found as a monomeric form. The mutants that have intermonomeric disulphide bridges between two subunit pairs (Avd-ci, Avd-cci) and the mutant that forms a continuous macromolecule (Avd-ccci) formed dimeric and tetrameric structures.

FIG. 2 shows the determination of binding of biotinylated alkaline phosphatase by wild-type avidin and the mutants Avd-ci and Avd-ccci after heat treatment (99.9 °C) for various periods of time by a microtiter plate assay.

FIG. 3 is a representation of heat-induced unfolding of avidin and the mutants without (A) and with (B) biotin in a baseline subtracted form.

DETAILED DESCRIPTION OF THE INVENTION

Avidin and streptavidin are valuable and widely used tools in the life sciences. In addition to their high biotin-binding affinity, the robustness and the flexibility of the system relies on their extreme stability under various demanding conditions. In the present application the object was to increase the stability of chicken avidin even further without losing its strong biotin-binding ability. In order to achieve this goal two, four or six intermonomeric disulphide bridges were introduced to avidin. In addition, the role of the intramonomeric disulphide bridges in the stability of the avidin tetramer was determined, by removing them using site-directed mutagenesis.

According to the DSC results, both the intramonomeric and intermonomeric disulphide bridges had effects on the heat-induced denaturation of avidin. Removal of the intramonomeric disulphide bridges from avidin (Avd-nc) caused a decrease in its T_m in the absence of biotin ($\Delta T_m = -8.9$ °C), whereas in the presence of biotin the T_m was virtually the same as that of the wild-type avidin. This underlines the importance of the biotin-induced increase in the stability of avidin. Interestingly, the cysteineless mutant Avd-nc has a slightly higher Tm than that of streptavidin as an apoform and significantly higher as a complex with biotin. Further, avidin has naturally higher Tm than streptavidin.

The most stable of the proteins is Avd-ci, which has an intermonomeric disulphide bridge in monomer interfaces 1-3 and 2-4. In contrast, the disulphide bridges created in interfaces 1-4 and 2-3 did not improve the thermal stability of avidin. Instead these mutations caused a decrease in the T_m of the mutant Avd-cci when compared to the wild-type avidin or even to the cysteineless mutant Avd-nc. This effect was more prominent in the absence of biotin. The high thermal stability of the combined mutant Avd-ccci indicated that the stabilizing effect of the disulphide bridge in interface 1-3 (and 2-4) exceeded the decrease caused by the disulphide bridges in interface 1-4 (and 2-3).

In the presence of denaturing agents it may be preferable to use the mutants Avd-ci, Avd-cci and Avd-ccci instead of wild-type avidin. This may particularly be the case for Avd-ccci since all of its monomers are covalently linked to each other (directly or indirectly). The mutant is capable of remaining as a tetramer even after unfolding, as seen in the SDS-PAGE analysis (Fig. 1B). Therefore it could be beneficial in applications where a leakage of subunits from matrix-coupled avidin tetramers would impede the qualitative and quantitative analysis of molecules. According to the DSC and microtiter plate assays, the avidin mutants Avd-ci and Avd-ccci, which had remarkably high T_m values even in the absence of biotin, could be utilized in PCR-protocols, because they can withstand the temperatures used to denature dsDNA. For example, the extraction of 2-iminobiotinylated or biotinylated ssDNA molecules at 95 °C is possible with Avd-ci and Avd-ccci coated particles.

In most applications of avidin-biotin technology high affinity biotin binding is essential. Consequently, all of the mutants introduced in this application are promising candidates for use with the methodology, as they all showed irreversible biotin binding and high affinity towards 2-iminobiotin. None of the mutations involved residues directly responsible for biotin binding. However, cysteine 106 that substituted isoleucine 106 in mutants Avd-cci and Avd-ccci, is located in the same loop (between β-strands 7 and 8) as the important biotin-binding residue tryptophan 110. The disulphide bridge formed between Cys106 of subunit 1 and Cys86 of subunit 4 may directly or indirectly have an influence on Trp110, and thereby also affect the biotin binding ability of these mutants.

It was also found that the streptavidin tetramer is unstable in certain conditions without its bound ligand. This could be detrimental in many applications due to the loss of signal along with the analytical molecules. Furthermore, it would presumably shorten the life span of materials if they were to contain covalently linked streptavidin molecules. Chilkoti as well as Reznik have described a streptavidin mutant, His127Cys11,12. This mutant has an intermonomeric disulphide bridge

His127Cys11,12. This mutant has an intermonomeric disulphide bridge between subunits 1 and 3 (2 and 4), which is analogous to the isoleucine 117 to cysteine substitution in our avidin mutant Avd-ci. They reported that the resultant mutant was more stable than wild type streptavidin (Reznik, G.O. et al., Nat Biotechnol 14 (1996), 1007-11. Avidin is, however, more stable than streptavidin, as judged by DSC analysis (Gonzalez, M. et al., Biomol Eng 16 (1999) 67-72). Therefore, the 1-3 (and 2-4) intermonomeric disulphide bridges bearing mutant Avd-ci would also have a higher Tm than the corresponding streptavidin mutant.

The production of streptavidin (or its mutants) is usually performed as inclusion bodies in E. coli. Its downstream processing includes laborious and time-consuming denaturation and renaturation procedures followed by the contrived formation of disulphide bridges (as in the case of the His127Cys mutant) and additional purification steps. In contrast, according to the present invention, the production of avidin mutants, even those with intermonomeric disulphide bridges, yielded soluble proteins in insect cells that were easily purified in a single step by affinity chromatography on a 2-iminobiotin column.

The avidin mutants include a signal sequence and thus are introduced in a eucaryotic insect cell to a special path, where building up of the disulphide bridges is possible as part of the process. The avidin mutants are thus soluble and functional immediately after this process. In this special path the bridging is enhanced by i.a. PDI (Protein Disulphide Isomerase) enzymes. On the contrary the streptavidin mutants are produced in an inactive form in procaryotic bacteria (US patent 6,022,951). Streptavidin has not been succesfully produced to a special path in an insect cell/baculovirus system, which system has now succesfully been used in avidin and avidin mutant production. Avidin also has considerably higher solubility than streptavidin.

Previously it was thought that because avidin already has one natural intramonomeric disulfide bridge, introducing new intermonomeric disulphide bridges would cause incorrect pairing leading to low quality or inactive protein in the cells. Streptavidin does not have a natural cysteine and thus this would not be a problem in streptavidin production.

Avidin and streptavidin are totally different proteins even though they have a similar structure. The mutant avidin Avd-ccci differs from the known avidin in that all the subunits are covalently attached to each other. It includes in addition to I117C also changes where intermonomeric bridges are formed by amino acids in different posi-

tions of the subunits, i.e. cysteine 86 pairs with cysteine 106 and vice versa (1-4 monomers and 2-3 monomers) and thus differs from the I117C mutant (or H127C streptavidin mutant), wherein the same amino acid in the pairing subunits are bridged in the disulphide bridging. Several disulphide bridges have not been introduced in a streptavidin tetramer than could be achieved by H127C, and such streptavidin mutant could be non-functional and presumably very heterogeneous.

When the side chains of isoleucines 117 in each avidin tetramer subunit are substituted with cysteines they form the 1-3 (and 2-4) disulphide bridges in mutants Avd-ci and Avd-ccci. Each subunit has isoleucine 106 and aspartate 86, in mutants Avd-cci and Avd-ccci these are substituted with cysteines in order to form 1-4 (and 2-3) intermonomeric disulphide bridges.

It is shown in this application that the intramonomeric disulphide bridges of wild-type avidin seem to be an important factor in making avidin so thermostable. On the other hand, it is possible to enhance the high thermostability of avidin even further by the introduction of intermonomeric disulphide bridges. The most thermostable avidin mutants described in this application provide more stable tools for avidin-biotin technology, suitable also for new kinds of applications.

The invention will be further described with reference to the following non-limiting examples.

Example 1.

Design of the mutants

Mutations were designed by using the sequence and structure information obtained from analyses with GCG (Genetics Computer Group, Madison, Wisconsin), EMBOSS (European Molecular Biology Open Software Suite), WHAT IF (Vriend, G., J. Mol. Graph. 8 (1990), 52-6, 29) and InsightII (Molecular Simulations Inc., San Diego, CA) programs. Genetic engineering of the coding sequence of avidin was performed by megaprimer (Sarkar, G. & Sommer, S.S., Biotechniques 8 (1990), 404-407) and QuikChange (Stratagene) methods, by using oligonucleotide primers containing the desired mutations.

The avidin mutant Avd-nc (C4A, C83Y) was constructed to obtain information about the importance of the intrinsic disulphide bridges to the overall stability of the avidin tetramer. According to the sequence alignment with streptavidin, the cysteine residues were substituted with the same residues that streptavidin bears in the

analogous positions in its primary structure (Table 1). The avidin mutant Avd-cci (D86C, I106C) adopted also the cysteines using the evolutionary approach (Laitinen, O.H. et al., FEBS Lett 461 (1999), 52-8). The sequence information came from the avidin-like domain of the sea urchin fibropellins (Hunt, L.T. & Barker, W.C., Faseb 3 (1989), 1760-1764; Delgadillo-Reynoso, M.G. et al., J Mol Evol 29 (1989), 314-27; Bisgrove, B.W. et al., Journal of Molecular Evolution 41 (1995), 34-45; Bisgrove, B.W. & Raff, R.A., Dev Biol 157 (1993), 526-38. In the case of Avd-cci, two intermonomeric disulphide bridges, designed to form between monomers 1-4 and 2-3 were introduced, thereby constituting a total of four new disulphide bridges per tetramer (Fig. 1A). In Avd-cci cysteine 86 from subunit 1 was presumed to pair with cysteine 106 from the adjacent subunit 4 and vice versa. Identical contacts were assumed to be present on the interface of subunits 2 and 3 as well.

Table 1. Description of the proteins used in this study. Number of intermonomeric disulphide bridges and the measured affinity towards 2-iminobiotin are indicated. Superscript (*) denotes that Avd-nc has no cysteines due to the removal of the wild-type intramonomeric disulphide bridge.

Sample	Mutated residues	No. of intermonomeric disulphide bridges	2-iminobiotin affinity K_d (M)
wt Avidin	None	0	2.5×10^{-8}
Avd-nc	C4A, C83Y	0*	1.9×10^{-7}
Avd-ci	I117C	2	8.5×10^{-8}
Avd-cci	D86C, I106C	4	9.4×10^{-8}
Avd-ccci	D86C, I106C, I117C	6	2.9×10^{-7}

Avd-ci (I117C) has an extra cysteine residue in each subunit of the tetramer. It was designed according to the mutational strategy that Chilkoti (Chilkoti, A. et al., Bio/Technology 13 (1995), 1198-1204) and Reznik (Reznik, G.O. et al., Nat Biotechnol 14 (1996), 1007-11) used to stabilize the streptavidin tetramer. In Avd-ci, cysteine 117 from subunit 1 faces cysteine 117 from the neighboring subunit 3. Identically, the corresponding subunit interface 2-4 of the wild-type protein contains two equivalent isoleucine residues, which were replaced by cysteines. Therefore, two intermonomeric disulphide bridges in the avidin tetramer were expected to form between subunits 1-3 and 2-4, intensifying the firm association between dimers 1-4 and 2-3 by the addition of two covalent bonds. Finally, in order to introduce six in-

termonomeric disulphide bridges into the avidin tetramer, Avd-ccci, a combination of mutants Avd-ci and Avd-cci, was constructed.

Example 2

Production, purification and characterization of mutant avidins

All the mutants were produced by a baculovirus expression system (Bac-To-Bac, Gibco BRL, Life Technologies, Gaithersburg, MD, USA) in the infected insect cells and purified by affinity chromatography on 2-iminobiotin agarose as previously described in detail by Airenne (Airenne, K.J. et al., Protein Expression and Purification 9(1997), 100-108) and Laitinen (Laitinen, O.H. et al., Biochem J. 363 (2002), 609-17).

Wild-type avidin was purified from chicken egg-white. Using a Vibra cellTM sonicator, the egg-white was sonicated for 3 minutes on ice at power setting 8 and 50 % duty cycle with a one-minute break between bursts. After sonication the sample was diluted with two volumes of PBS and centrifuged (20 minutes, 20.000 g, 4 °C). The soluble fraction was further purified by affinity chromatography on 2-iminobiotin agarose as previously reported (Laitinen, O.H. et al., J Biol Chem 276 (2001), 8219-24). Protein samples were concentrated and subjected to change of buffers with Centricon YM-3 filters (Millipore, cat. no. 4202). The SDS-PAGE analysis was performed with a sample buffer without β-mercaptoethanol.

All the mutants showed excellent purification efficiency, indicating that the mutations had no major effects on the 2-iminobiotin-binding properties. Moreover, the mutants showed irreversible biotin-binding properties indistinguishable from that of wild-type avidin as measured with an IAsys optical biosensor (data not shown). Affinities towards 2-iminobiotin were determined for wild-type avidin and the four mutants (Table 1). The results indicated that the mutations had not significantly altered the 2-iminobiotin binding-characteristics of the mutants. The formation of the intermonomeric disulphide bridges was studied with SDS-PAGE analysis (Fig. 1B), which confirmed that the cysteines formed pairs in the manner expected. The biotin-binding activity after heat treatment of Avd-ci and Avd-ccci was studied by a microtiter plate assay (Fig. 2). The mutants proved to be more stable and they remained active, in the sense of binding biotin, longer than the wild-type avidin.

Example 3.

Biotin-binding assays

Reversibility of biotin binding was measured for avidin and the mutants with an IAsys optical biosensor (Laitinen, O.H. et al., FEBS Lett 461 (1999), 52-8). Protein samples were allowed to bind to a biotin-aminosilane cuvette in PBS containing 1 M NaCl. After the equilibrium was reached, biotin-containing buffer was added and the dissociation of the proteins was monitored. The affinities of the proteins towards 2-iminobiotin were determined with an IAsys optical biosensor (Marttila, A.T. et al. FEBS Lett 441 (1998), 313-7). Biotin-binding activity of the mutants Avd-ci and Avd-ccci after heat treatment was studied with a microtiter plate assay. Protein samples, 5 µg / ml concentration in PBS, were heated for various time periods at 99.9 °C and then chilled on ice. The samples were transferred to a Nunc Maxisorp-plate and incubated at 37 °C for 2 h. The wells were washed three times with PBS-Tween (0.05 % v / v). After that the wells were blocked with PBS containing 1% BSA at 37 °C for 30 minutes and washed again with PBS-Tween. Next, biotinylated alkaline phosphatase (Sigma) in PBS, 1% BSA was added and incubated at 37 °C for one hour. Once again, the wells were washed with PBS-Tween and the PNPP substrate in DEA buffer (1 mg / ml) was applied to the wells. The absorbances at 405 nm were measured after incubation for 45 minutes.

Example 4

Differential scanning calorimetry

In order to study the thermal stability of the purified avidin mutants, they were subjected to analysis by differential scanning calorimetry. A Nano II differential scanning calorimeter (Calorimetric Science Corporation, Provo, Utah) was used. The protein sample concentrations were 0.032 mM (given as the monomer concentration) and the biotin-containing samples had a biotin:avidin molar ratio of 3:1. The reference cell was filled with the same buffer that the sample proteins were dissolved in (100 mM Na-phosphate buffer pH 7.4). The thermograms were recorded as a function of temperature, between 25 and 130 °C at a temperature scan rate of 55.6 °C/h. The baselines were subtracted and the transition midpoints (T_m) of the samples were calculated using proprietary software provided by the instrument manufacturer.

The results are shown in a baseline-subtracted form (Fig. 3) and numerically (Table 2). As shown by the thermograms, Avd-ci was the most stable protein since its de-

naturation Tm value was the highest, both in the absence and presence of biotin. Avd-ccci was only slightly less stable than Avd-ci. The other mutants and wt avidin were equally stable when bound to biotin, whereas Avd-nc and Avd-cci showed the lowest Tm values in the absence of biotin. The heat-induced unfolding of the avidins was an irreversible process and the unfolding of the proteins was often followed by a more or less sharp decrease in the heat capacity (due to aggregation, data not shown).

Table 2. Heat-induced unfolding of the wild-type and mutant avidins with or without biotin was examined using DSC. Values for T_m are the averages of the results from two different experiments (± S.E.M.). The value for ΔT_m indicates the difference in Tm compared to that of wt avidin or wt avidin + biotin.

Sample	T_m/(°C)	ΔT_m/(°C)
wt Avidin	85.5 ± 0.1	
+ biotin	118.2 ± 0.1	
Avd-nc	76.4 ± 0.4	-9.1
+ biotin	118.5 ± 0.1	0.3
Avd-ci	98.6 ± 0.1	13.1
+ biotin	124.7 ± 0.5	6.5
Avd-cci	74.4 ± 0.1	-11.1
+ biotin	117.5 ± 0.1	-0.7
Avd-ccci	94.7 ± 0.5	9.2
+ biotin	122.0 ± 0.1	3.8

The invention has been illustrated by examples and embodiments, but it may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention and all such modifications are intended to be included within the scope of the enclosed claims.

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Claims

1. A thermostable avidin mutant having intermonomeric disulphide bridges in the tetramer.

2. A thermostable avidin mutant of claim 1 wherein the tetramer has one disulphide bridge between monomers 1-3 and one disulphide bridge between monomers 2-4.

3. A thermostable avidin mutant of claim 1 wherein the tetramer has two disulphide bridges between monomers 1-4 and two disulphide bridges between monomers 2-3.

4. A thermostable avidin mutant of claim 1 wherein the tetramer has one disulphide bridge between monomers 1-3 and one disulphide bridge between monomers 2-4 and two disulphide bridges between monomers 1-4 and two disulphide bridges between monomers 2-3.

5. A thermostable avidin mutant of claim 2 wherein the isoleucine residue 117 has been changed to cysteine.

6. A thermostable avidin mutant of claim 3 wherein the isoleucine residue 106 has been changed to cysteine and aspartate residue 86 has been changed to cysteine.

7. A thermostable avidin mutant of claim 4 wherein the isoleucine residue 117 has been changed to cysteine, the isoleucine residue 106 has been changed to cysteine and aspartate residue 86 has been changed to cysteine.

8. A method for stabilizing avidin by introducing disulphide bridges between its subunits.

9. A method of claim 8, wherein baculovirus expression system is used in insect cells and soluble proteins are purified by affinity chromatography.

10. A method of claim 9, wherein a 2-iminobiotin column is used in the affinity chromatography.

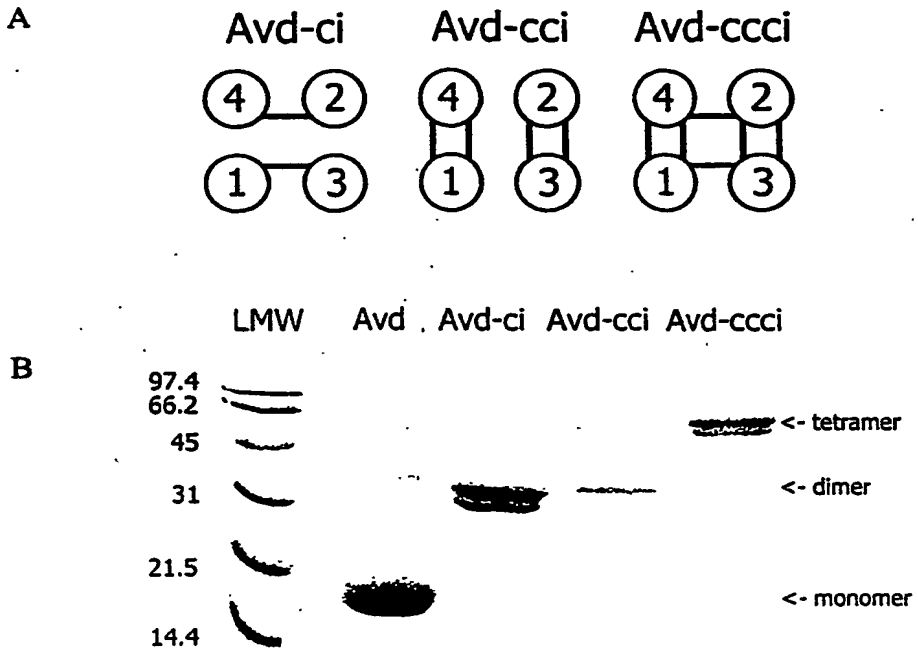
L3

Abstract

The present invention relates to a method for stabilizing avidin by introducing disulphide bridges between its subunits and to the thermostable avidin mutants produced. These new avidin mutants will find use in applications where extreme stability or minimal leakage of subunits is required.

Fig. 1

L4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Avd-ci
Avd-cci
Avd-ccci
4
2
1
3
B
LMW
Avd
Avd-ci
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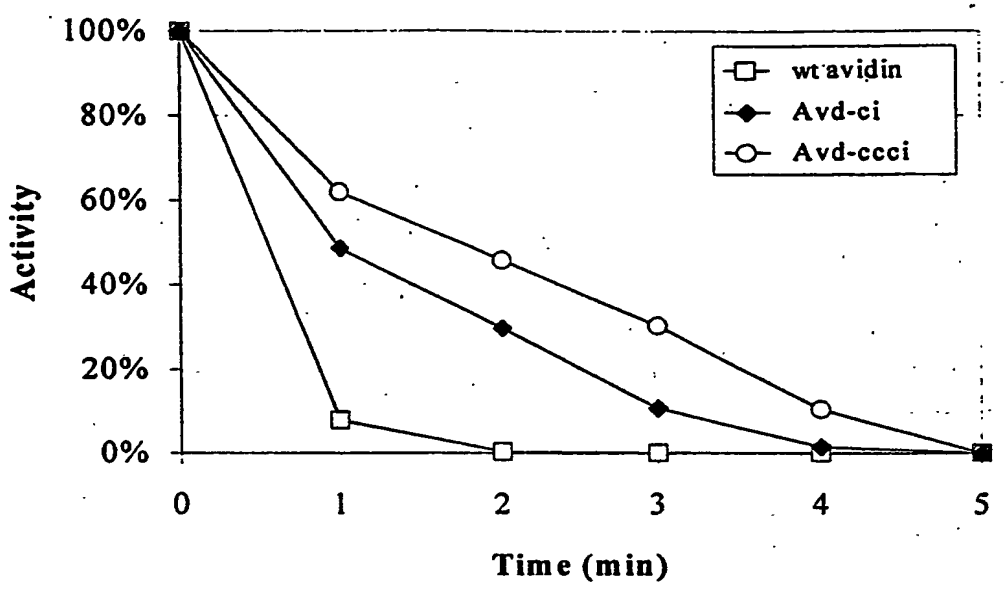
66.2
45
31
21.5
14.4
<- tetramer
<- dimer
<- monomer

Figure 1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60%
40%
20%
0%
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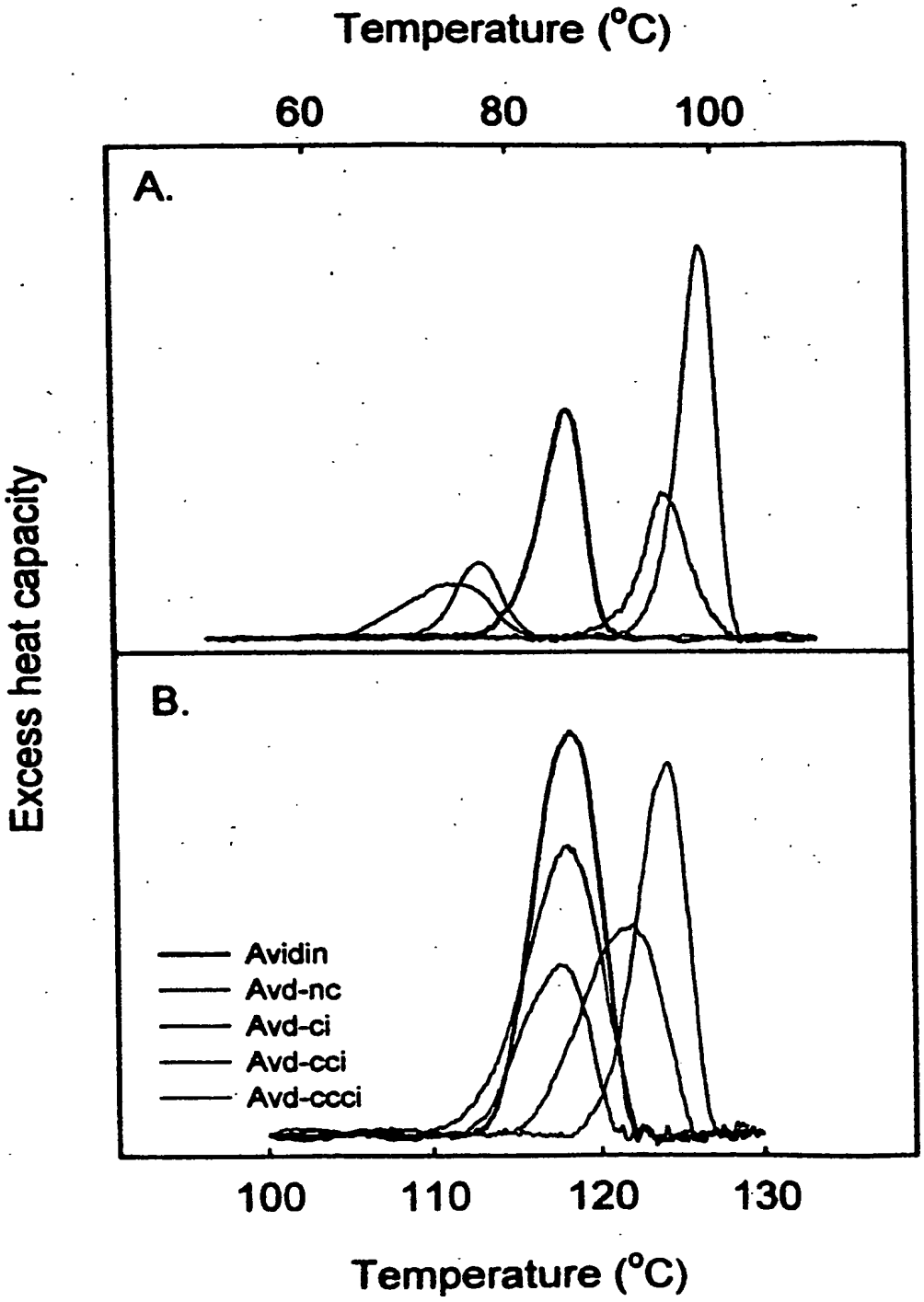
2
3
4
5
Time (min)
wt avidin
Avd-ci
Avd-ccci

Figure 2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80
100
A.
B.
Excess heat capacity
Avidin
Avd-nc
Avd-ci
Avd-cci
Avd-ccci
100
110
120
130
Temperature (°C)

Figure 3